Survival Kinetics of Cultured Human Lymphoma Cells Exposed to Adriamycin

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SUMMARY

Cultured human lymphoma cells exposed to adriamycin exhibited extreme sensitivity to the drug. When these cells were exposed to adriamycin for 12 hr, a 5-log kill in terms of reproductive capacity was observed. Furthermore, under these conditions, adriamycin exerted an immediate (interphase) lethal effect on about 60% of the cells. Synchronized cells revealed that the S-G2 boundary region was the most sensitive stage of the cell cycle. Insofar as adriamycin can achieve a great killing effect at low doses and with short exposure times, we suggest that a superior clinical therapeutic index could be achieved by utilizing adequately spaced low doses of adriamycin in the treatment of human tumors.

INTRODUCTION

Adriamycin is a new antitumor antibiotic of the anthracycline group isolated from Streptomyces peucetius var. caesius (1). Adriamycin differs structurally from daunomycin only in the substitution of a hydrogen atom by a hydroxyl group on the acetyl radical of the aglycone moiety (8). In spite of such a minor difference, adriamycin shows a much higher therapeutic index than daunomycin in experimental animal tumors (8). Furthermore, adriamycin has an increased retention time in the body (7) and a wider range of action in human cancers (3). Adriamycin quickly penetrates into the cells and fixes to the nuclear structures with a marked localization at the perinucleolar chromatin (21). It is considered that adriamycin, like its analog daunomycin, intercalates with DNA (5), thus inhibiting not only DNA-dependent RNA synthesis (27) but also DNA duplication by affecting template-related DNA polymerase activity (25). Chromosomal abnormalities are rapidly induced with extremely low concentrations of adriamycin (24), and the drug has also shown immunosuppressive activity (6, 13, 16). Clinical trials with adriamycin have been carried out in several centers, and its toxicity and dose range have been established (17, 26), but only recently have some reports on the effects of adriamycin at the cellular level appeared in the literature (2, 14).

This paper describes the effect of adriamycin on cellular survival of human lymphoma cells cultured in vitro.

MATERIALS AND METHODS

Drug. Adriamycin (14-hydroxydaunorubicin), manufactured for clinical experimental use (Soc. Farmaceutici Italia An. p. Az, Milan, Italy), was used in these experiments. Adriamycin solutions in 0.9% NaCl solution were always prepared immediately before they were dispensed into the growth medium. The pH ranged from 7.2 to 7.4.

Cell Culture Techniques. Monolayer cultures of T1 cells, a human lymphoma cell line (22), were maintained in Ham's F-10 medium supplemented with 20% fetal calf serum, vitamins, glutamine, and antibiotics. Under these conditions, the average cell-cycle time was 27 hr. The pre-DNA synthesis period (G1) was 3.5 hr, the DNA synthesis period (S) was 13.5 hr, and the post-DNA synthesis period (G2) was 10 hr. Experiments involving asynchronous populations were performed on cells in logarithmic growth. Synchronized S-phase cells were obtained by a single treatment with 3 mM TdR2 as previously described (10). The degree of synchrony was monitored by 30-min pulse labeling with 1 μCi of TdR,3H per ml, to determine the percentage of cells in S phase, and by scoring the MI. At the end of the synchrony procedure, 90% of the cells were in S phase and moved synchronously into G2 (80%). After a mitotic peak of 11 to 14% (the MI of asynchronous cells is 1.6), the cells were still partially synchronized in G1 (70 to 80%). To increase the yield of G1-phase cells, a single block with TdR of 24-hr duration was followed by mitotic selection at the time calculated for the arrival of the cells at the end of G2. This technique provided up to 90% of cells in mitosis, which cells promptly entered into G1 phase after incubation at 37°C. These procedures did not significantly alter the plating efficiency of T1 cells. In all radioautographic procedures, a 50% solution of Ilford K5 emulsion in distilled water was used. The radiolabeled cells were exposed for 1 to 2 weeks and developed in Kodak D19 developer. To assess survival, we harvested treated and control cells growing in 60-mm Petri dishes by standard procedures (12) and counted them with the aid of an electronic particle counter (Coulter Electronics, Hialeah, Fla.). Known aliquots were dispensed into 60-mm Petri dishes so that 50 to 100 colonies would appear after 21 days incubation in a 5% CO2 humidified atmosphere at 37°C. The colonies were stained with 0.5% crystal violet in 95% ethanol. Viability was defined as the

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2The abbreviations used are: TdR, thymidine; MI, mitotic index; LI, labeling index.
ability of single cells to give rise to a colony of 50 or more cells. In each experiment, at least 6 plating-efficiency controls were run in parallel. The survival fractions of the dose points were normalized with respect to the individual controls for each experiment. In this series of experiments, the plating efficiency ranged up to 40%, and the coefficient of variation never exceeded 10% at each dose point.

In experiments designed to test the effects of adriamycin as a function of duration of treatment, both the supernatant medium of treated cells and the monolayer cells were pooled together after harvesting, since it was noticed that adriamycin rapidly detached many cells from the monolayer. In addition, we processed an aliquot by the trypan blue exclusion technique, as previously described (12), to assess immediate (interphase) cellular death.

RESULTS

Dose-Response Survival of Asynchronous T1 Cells. The survival of asynchronous T1 cells treated with adriamycin for 1 hr is shown in Chart 1. The survival is an exponential function of the dose with no shoulder region. The curve is characterized by a $D_0$ of 0.15 $\mu$g/ml (1 hr) and an extrapolation number, $n$, of 1.

Survival as a Function of Duration of Treatment. The colony-forming capacity of T1 cells treated with a single dose of 0.1 $\mu$g of adriamycin per ml decreased steadily as a function of the duration of exposure to the drug (Chart 2). After 12 hr of treatment, reproductive survival had been reduced 100,000-fold (5 log decades). The drug also had an immediate effect on the metabolic viability of the cells. Although T1 cells are usually firmly attached to the culture vessel, they rapidly detached from the monolayer after treatment with adriamycin. Thus, from an occasional floating cell in control cultures, the ratio of floating to monolayer cells became 0.5 after 12 hr of treatment. Furthermore, while the majority of control cells were able to exclude trypan blue, almost two-thirds of the treated population showed dye incorporation after 12 hr (Table 1).

Survival Response of Synchronized Cells. Synchronization in S and G2 was achieved with a single TdR block. This provided 90% cells in S phase, as judged by the LI, and about 80% of cells in G2 calculated from the LI and MI. The percentage of G1 cells obtained by this technique was similarly estimated at 70 to 80%. In other experiments, a high yield of G1 cells was obtained by the combination of TdR block and mitotic selection. The results in these last experiments were similar to those obtained with G1 cells resulting from the single-TdR-block technique with no mitotic selection. Thus, the lower degree of synchrony obtained with the latter technique did not significantly alter the survival data. Therefore, we considered both sets of data together in constructing the relative sensitivity of G1 cells to adriamycin. Chart 3 depicts the relative sensitivity of synchronized T1 cells to a single dose of 0.5 $\mu$g of adriamycin per ml for 1 hr at selected points in the cell cycle. The curve indicates
fluctuations in the survival of reproductive capacity from a minimum in the S/G2 boundary to a 10-fold-increase maximum in late G1. S-phase cells exhibit moderate sensitivity, whereas G2-phase cells apparently exhibit the greatest sensitivity to adriamycin. Dose-response curves of synchronized cells at appropriate times are depicted in Chart 4. All of the curves show a straight exponential decrease in survival as a function of the concentration of adriamycin given for 1 hr. There is no shoulder region, and all of the curves are characterized by an n value of 1. The various D0’s are as follows: early S, 0.13 µg/ml; mid-S, 0.17 µg/ml; S-G2 boundary, 0.09 µg/ml; G2, 0.08 µg/ml; early G1, 0.13 µg/ml; and G1-S boundary, 0.19 µg/ml.

DISCUSSION

Direct quantitative extrapolation to clinical dosage from in vitro data is precarious. Therefore, it is interesting to compare the effects of doses of different drugs on a single target-cell line. This can be done by contrasting the shape of the respective survival curves (i.e., continuous exponential decrease in survival with increasing concentrations of drug; asymptotic curves reaching a plateau of no killing effect beyond a given concentration; and the presence of a shoulder region quantified by an extrapolation number, n, resulting from the intercept of the linear part of the curve and the ordinate, etc.) and by direct comparison of the slopes of linear curves considering the D0, the inverse of the slope, which is defined as the mean lethal dose. Table 2 compares the D0 (1 hr) of adriamycin on asynchronous T, cells with the D0’s of other drugs studied on the same lymphoma cell line. It can be seen that adriamycin is an extremely effective drug in reducing the survival of T, cells at low doses and in short exposures with no visible shoulder region of no killing effect. This marked effect (D0 = 0.15 µg/ml, 1 hr) of adriamycin of T, cells parallels its effect on Chinese hamster cells (D0 = 0.2 µg/ml, 1 hr) (2) and on HeLa cells (14).

Razek et al. (19) recently reported that adriamycin has a marked cytocidal action in both leukemic and hematopoietic colony-forming units in mice. However, their survival curves presented a shoulder region of no killing effect, and they suggested repair of sublethal damage induced by adriamycin as a possible explanation for this phenomenon. We have not found a shoulder zone in our survival curves obtained in vitro, nor have shoulders been noted in CHO cells by Barranco et al. (2) or in HeLa cells by Kim and Kim (14). In vitro studies investigate directly the effects of drugs at the cellular level. The main assumption for in vitro studies is that the response of cultured cells can mimic that of their in vivo counterpart once the drug has reached the neoplastic elements. It has already been shown (15) that such correspondence may exist. With in vivo systems, such as those utilized by Razek et al., in which the drug is injected into the host, one has to contend with pharmacological aspects of drug absorption, binding, excretion, distribution, and metabolism (18) before defining the effects of a drug on the target cells. Therefore, it is possible that the shoulder region described by Razek et al. is a pharmacological “artifact” and does not correspond to any repair system in the target cells.

The fact that, at low doses and after short exposures, adriamycin is a potent cell killer, has great clinical relevance. One of the most important untoward effects of adriamycin is its cardiac toxicity, which appears to be cumulative, and the maximum total dose of adriamycin must be limited (17, 26). Since low doses of adriamycin can conceivably reduce the actively proliferating tumor cell population by several decades,
it seems that a much greater therapeutic index3 could be achieved by utilizing low dose pulses of adriamycin at variable intervals. These intervals would be based on knowledge of the proliferation kinetics of the neoplastic population. Although it is possible that adriamycin may affect resting-phase cells (G₀), its efficacy on proliferating cells leaves no doubt. Therefore, a single treatment with a low dose of adriamycin should reduce the proliferating population to a minimal expression. Additional immediate therapy would not significantly reduce the tumor mass. Another pulse with low doses of adriamycin could then be given when sufficient cells have abandoned the G₀ compartment and have commenced proliferation. Preliminary clinical studies have indicated that schedule manipulation of adriamycin chemotherapy does not result in greater efficacy (20, 23), but it is conceivable that therapeutic gain can be obtained by reducing the cumulative toxic effects of the drug. Tumor response to adriamycin apparently lasts only as long as therapy continues, but marked cardiac toxicity limits the total dose of adriamycin tolerated. If a lower dose is as effective as a larger one, longer duration of response could be anticipated with the lower-dose regimen, emphasizing the clinical importance of this inference, if it is proved valid. This hypothesis is currently under investigation by the Southwest Cancer Chemotherapy Study Group, with a clinical protocol designed to test the hypothesis. If it is validated, this treatment strategy may improve the clinical importance of adriamycin chemotherapy.

In experiments involving time duration effects, a 12-hr treatment with low concentrations of adriamycin (0.1 µg/ml) lowered the survival by 5 log decades. This indicates that the killing effect of the drug is not limited by stage sensitivities or the presence of a resistant population in T₁ cells. However, low-dose continuous infusion may not be a desirable clinical procedure, since adriamycin causes great damage to vascular endothelium. Adriamycin also exerts an immediate lethal effect on cellular metabolic integrity, as was demonstrated by dye-exclusion tests, which could indicate an effect on nonproliferating cells. Adriamycin-treated cells showed fluctuations in survival depending on their location in the cell cycle. In experiments involving synchronized cells there is always some degree of uncertainty about the degree of synchronization achieved by the synchronizing techniques. However, in our system, and judging by the temporal markers used (L1 and M1), there was a substantial accumulation of phased cells in the various stages of the cell cycle. T₁ cells exhibited maximal sensitivity to adriamycin in the boundary region between S and G₂ and exhibited least sensitivity in G₁. These results differ from those reported by Kim and Kim and by Barranco et al. in HeLa and CHO cells, respectively. In their systems, the DNA synthesis period was the stage of greatest sensitivity. It is apparent that adriamycin may present different stage sensitivities depending on the cell line. The phase of the cell cycle during which daunomycin, the analog drug, exhibits its maximal inhibitory effect on RNA synthesis also differs in different cell lines (4, 28). In spite of these fluctuations in activity, adriamycin can hardly be considered clinically a phase-sensitive drug. As has already been indicated by Barranco et al. (2), the continuous dose-dependent logarithmic killing of adriamycin in asynchronous cells evidences that adriamycin is extremely efficient at killing all proliferating cells, no matter where they are in the cycle at the time of treatment. Furthermore, in our system, adriamycin had a 5 log killing effect in only 12 hr less than one-half the time of the cell cycle, again indicating no stage-sensitivity constraint.

The data reported in this paper indicate that adriamycin is a valuable addition to the cancer chemotherapy arsenal. The data also demonstrate the usefulness of cellular pharmacological studies in relation to the planning of clinical chemotherapeutic protocols.

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